THE PRIMARY SEQUENCE OF ACETYLCHOLINESTERASE AND SELECTIVE ANTIBODIES FOR THE DETECTION OF ORGANOPHOSPHATE TOXICITY

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SUMMARY

The proposed work has been directed to determination of the structure of acetylcholinesterase. During the project period we have determined the primary structure of the Torpedo enzyme through amino acid sequencing and the isolation and sequencing of a cDNA clone encoding for the 11S form of the enzyme. The recombinant DNA studies involving the isolation of cDNA clones encoding acetylcholinesterase were part of studies supported by an NIH grant. However, they relied on amino acid sequence information for preparation of oligonucleotides for screening and ultimately yielded complementary information on primary structure. Peptides corresponding to the active center of the enzyme and a C-terminal region have been synthesized and antibodies are being raised for the purpose of detecting the phosphorylated enzyme and delineating functional regions of the molecule. Finally, the disulfide bonding pattern has been determined for the 11S form of the enzyme.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

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Introduction

During this period we have completed the primary structure of acetylcholinesterase from Torpedo californica through amino acid sequencing and cDNA cloning and sequencing. This information should prove important to the many groups working on acetylcholinesterase structure.

Background

The high turnover number of acetylcholinesterase and the availability of selective inhibitors have allowed extensive study of its distribution, catalytic mechanism, and functional role in cholinergic neurotransmission.
The recent elucidation of the primary structure of the enzyme through protein chemistry and the isolation of its gene have added a molecular dimension to continuing studies on this protein, which controls the residence time of acetylcholine in the synapse. Acetylcholinesterase exhibits an extensive polymorphism of structure and, since the catalytic parameters of the individual enzyme species are largely invariant, the structural diversity appears critical only to the regulation of the cellular disposition of this molecule. Recent structural studies clearly show that acetylcholinesterase behaves as a secreted rather than an integral membrane protein. The posttranslational modifications provide an appropriate link to tether the enzyme to specific extracellular locations. Since acetylcholinesterase is an extracellular enzyme, modifications of structure critical to its disposition should occur prior to export to its site of residence. Thus variations in structure responsible for cellular localization must either be encoded in the genome or be differentially affected by post-translational events of biosynthesis.

Acetylcholinesterase Polymorphism

Since the initial finding of Massoulié and Reiger (1) that a native form of acetylcholinesterase contains an elongated tail unit linked to a defined number of catalytic subunits, the control of individual species of acetylcholinesterase in relation to innervation, developmental processes and activity of excitable cells has received considerable attention (2). Two general classes of acetylcholinesterase species exist. The most unique is the elongated or dimensionally asymmetric species, which contains a filamentous tail unit disulfide-linked to tetrameric sets of catalytic subunits. The tail unit contains a collagen-like sequence distal to the catalytic subunits. Each strand of the triple helix is joined to a tetramer of catalytic subunits. Since each catalytic subunit is approximately 70,000 daltons, elongated species close to a molecular weight of 1 million are generated. In the case of Torpedo, but not Electrophorus, a second type of structural subunit has been identified as a noncollagenous, 100,000-dalton peptide (3). It will be of interest if this structural entity also prevails in higher species. Treatment of the asymmetric form with collagenase markedly shortens the tail unit and a light tryptic digestion will remove the structural subunits without apparently altering catalytic parameters or the structure of the catalytic subunit (cf. 2). The asymmetric species appear to be fully assembled in the Golgi apparatus prior to export from the cell (4,5).

The second class includes the globular forms, which show considerable structural variation in subunit assembly (monomers to tetramers) and in hydrophobicity. The hydrophobic forms identified to date result from the cotranslational addition of glycophospholipid to the C-terminal carboxyl group of the nascent peptide chain (6,7). This modification resembles that seen in the variable surface glycoprotein of trypanosomes and the Thy-1 antigen (8). It is quite possible that the natures of the glycophospholipid additions are not identical in the various tissues and may, in themselves, provide a basis for microscopic regional localization. Hence, the globular forms range from totally soluble species to species with particular hydrophobic glycophospholipids conjugated to the peptide chain.

Methods

The methods used for generation of the data described below have been documented in our manuscripts now published in the open literature (3,9-12) and will be described only briefly.

Our overall strategy was to sequence the catalytic subunits from the 11S and 5.6S forms of the enzyme. The primary peptides were obtained from a tryptic digest after disulfide bond reduction and carboxymethylation with iodoacetate. They were then size fractionated on Sephadex G-50. The individual fractions were categorized as I-X and then fractionated on reverse phase high pressure liquid chromatography (HPLC) using a C-18 column with 0.1% trifluoracetic acid (TFA) and usually a 0-50% gradient of acetonitrile. These peptides were then numbered by consecutive fractions and catalogued. In cases where fractionations were incomplete, fractions were then subjected to HPLC using a C-4 column or HPLC on C-18 or C-4 columns using a phosphate, pH 7.0 buffer. Details on the cataloging of peptides and their sequences may be found in the year-end report (year 2) (13). As noted below, a similar strategy was employed to obtain the disulfide linked peptides.

The enzymes were purified to homogeneity by affinity chromatographic procedures adopted in our laboratory many years ago (3). Each enzyme form was homogenous, as ascertained by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and by assay of specific activity.

1. Determination of the primary structure of acetylcholinesterase

Primary structure determinations relied on both tryptic and CNBr fragmentation. The peptides were initially size-separated on Sephadex G-50 and then subjected to reverse-phase HPLC on C-18 or C-4 columns. Sequencing initially involved the dansyl-Edman and the spinning cup methods, but after the first 6 months of the contract we employed the gas phase method (9). The changeover to the gas phase method followed our making the instrumentation operational and obtaining reliable and reproducible data on representative peptides. Amounts down to 50 pmol of peptide could be sequenced by the latter method. Our basic sequencing strategy for the tryptic peptides was to reduce and alkylate the protein with [^{14}C]iodoacetate and size fractions were collected which were then subjected to reverse-phase HPLC on C-4 columns, using an acetonitrile-1% aqueous TFA gradient. Peptides that fractionated poorly on C-18 columns usually resolved well on C-4 columns. Compositions and N-terminals were ascertained before subjecting the peptides to gas-phase sequencing. Cyanogenbromide (CNBr)

fractionations also involved reduction and alkylation with [^{14}C]-iodoacetate, treatment with CNBr and size fractionation on Sephadex prior to HPLC. For the CNBr peptides, fractionation on C-4 columns worked best. In some fractionations the active site peptide was labeled with [^{3}H]diisopropylfluorophosphate.

2. Antibody generation and assessment of reactivity

Both monoclonal and polyclonal antibodies were made to the 11S and 5.6S species of acetylcholinesterase. Similar methods were used for generation of antibodies to the individual peptides. Antibody reactivity and titers were determined by the enzyme-linked immunoassay (ELISA) method and by radioimmunoassay using [^{125}I]acetylcholinesterase (3,10).

3. Cloning and sequencing of a cDNA-clone encoding the 11S species of acetylcholinesterase

Nucleotide probes to tandem sequences contained within a single CNBr peptide were used to hybridize recombinants in a λ-gt 10 library (11). Positive clones were isolated, tested by hybridization and sequenced using M-13 sequencing vectors (11).

4. Determination of the disulfide bond linkages in the 11S species of acetylcholinesterase

Unreduced acetylcholinesterase was reacted with bromobimane to label free sulfhydryls. Tryptic and CNBr peptides were prepared and candidate disulfide peptides isolated by having unique positions on HPLC. These peptides were then reduced by dithrothreitol and alkylated with [^{14}C]iodoacetate. Upon chromatography, these peptides were isolated and sequenced (12).

Results

1. Amino acid sequencing of Torpedo acetylcholinesterase

Our sequencing strategy is designed to fulfill several objectives:

a. To obtain a sequence sufficient for the design of multiple nucleotide probes for cDNA library screening.

b. To employ the sequence to verify inferred amino acid sequence resulting from cDNA sequencing and correlate the cDNA sequences with the multiple acetylcholinesterase gene products.

c. To elucidate differences in sequence between the molecular forms of acetylcholinesterase.

d. To identify critical regions in the molecule: active center, chemically modified residues, N-terminal sequence, C-terminal sequence, glycosylation sites, cysteine-containing peptides and other sites of post-translational modification.

e. To provide a peptide fractionation scheme by which other cholinesterases of lower abundance can be sequenced and homologous regions identified.

To date, we have sequenced about 80% of the tryptic peptides of the 11S enzyme and 30% of the tryptic peptides of the 5.6S enzyme. All of the CNBr peptides have been isolated from the 11S enzyme and about 30% have been sequenced. A smaller number have been sequenced in the 5.6S enzyme. The sequences are summarized in Table I. Several findings should be highlighted:

a. Large tryptic peptides for the active center (24aa) were isolated and sequenced. Sequence was verified by chymotryptic digestion and the position of this peptide in the whole enzyme could later be verified. The active center serine is serine 200. These peptides are identical in the 11S and 5.6S species (1) (Fig. 1).

b. The N-terminal peptides of the 11S and 5.6S enzymes were identified and sequenced through 42 and 30 residues, respectively. These peptides were also identical in the 11S and 5.6S species. In some cases glutamine appeared at position 6 and asparagine at position 3, but this occurred in the minority of cases. These sequences were later verified by the cDNA sequence and, more important, a leader peptide was demonstrated for the unprocessed acetylcholinesterase. Cleavage occurred C-terminal to an Ala, giving rise to the N-terminal Asp residue in the processed protein. A candidate C-terminal tryptic peptide ending in leucine was also identified in the 11S species. That this peptide was a C-terminal tryptic peptide was later verified by finding a stop codon corresponding to amino acid position 575, which followed the leucine codon and thus ended the open reading frame on our cloned cDNA. An analogous C-terminal peptide has not been found for the 5.6S enzyme, and we believe a posttranslational modification occurred here, providing one of the points of structural departure of the two enzyme forms.

c. The cysteine-containing peptides were identified by reduction and subsequent alkylation by [^{14}C]iodoacetate. We obtained more cysteine peptides than would be predicted by the c-DNA sequence, but they arose simply from incomplete cleavages. All of these peptides can be placed in the inferred amino acid sequence on the basis of either their total sequence or their N-terminal residue identification and partial sequences. We have initiated fractionations of the unreduced enzyme with the essential aim of establishing the positions of the inter- and intrasubunit disulfide bridges. One of the eight cysteines appears to exist as a free sulfhydryl group and

Table I Sequences of Torpedo californica Acetylcholinesterase Peptides*

11S Acetylcholinesterase (tryptic peptides)	
I46	ivgywapfa-c
I?	vpvegcvfanef-nnci
III88	fsivpvddggfw(yst)k
II61	kpwsgvw-asnyp (carbohydrate and CM cysteine)
II61	kpwigvwfhnypl
IV33	dnhellvntksgkvmgtrvpvlsshisafl givfaeqvgidv (N-terminal)
IV67	tvtifgesaggasvgmhilspgsr (active site)
IV14	tgnpneptsqesk
IV26	le-ea
IV?	ailqsg-vdcepa
I?	ivgywaafa-c
I?	vpvegcvfanef-nnci
V?	vqvcwfwnqflp
VIII?	rpepk
V02	fidlntepmnk
V04	galqwvhdniqffggdpmk
I67	iteahh
II49	nlbbglncnl-nsaeelihicl
III65	-(av)dedcly-niwspgca
IV69	v-afalig
II67	vphandlgldv(g)lqytdwmddnngik
IV?	hescael (C-term)
II61	kpw(i)gvw-as(n)ypl (carbohydrate, CM cysteine)
I46	ivgywa2fa-(c)
I?7	v(p)vegcvfane(f)(lp)nnci
III88	f(s)ivpv(d)dgqfw(ystk)
II63	dglddivgbhnvicplmhf
II62	kpwphawdlg-p
II64	lsvphandlgldlvt
II101	dhnlvwpew-gvi(h)gyei--g-l-p
II68	lsvphandlgldtvgloytdwmd(ing) (4-E7 immunoreactive)
I?3	v(aph)vegcvfane(yf)(lp)(np)nc(f)-(hg)v(if)e
II49	nlbbglncnl-nsagglihicl (carbo)
III?	-(av)(sd)edcly-niw(s)pgca
IV?	ailqsgspncpwasvsv(aZg)p
VII?	vg(v)cxfwngflp
I61	kpw(l)gvw(f)(h)(n)y(p)l
I?3	v-vegcvfane--ncf--v(ip)g
III64	b dedcly-niw-pgc
V65	ailqsgspncpwasvsv(azg)r
V01	tgnpnephsqesk
II54	lgvp-a
IV64	aieag
IV71	tvtifg-s

11S Acetylcholinesterase (tryptic peptides cont'd)	
V187	vq-cwfwnqflp
VIII7	rpepk
V01	tgnpnephsqesk
V02	fidlntepmnk
V03	ailqsgspncpwasvsv
V04	galqwvhdniqffggdpmk
III65	--dedcly-niw-pgc
II61	pw-gv--a--vpl
IV26	hescael (C-terminal peptide)
I77	v-vegcvfane-nnci
II01	d-nlvwpew-gvi-gy
II49	dlbbglncnl-nsaeeli--cl
II59	lgvpda
II67	l-vphandlgldtvglqytdwmd
III88	f-ivpv-dgqfw
IV14	tgnpneptsqe
IV62	fgdgtyly
IV64	aieag
IV71	ivtifg-s
I46	ivgywa-fa
I67	iteah
IV69	v-afali

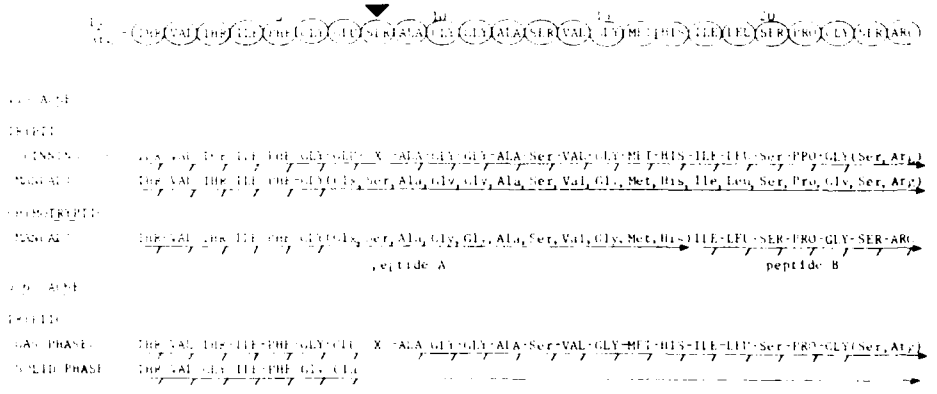
5.6S Acetylcholinesterase (tryptic peptide)	
IV18	tgnpnep
IX02	gpha-a
VI64	ail-e--pncpwwatv-va
	dnhqllvntksgkvmgt (N-terminal)
	tvtifgesaggasvgmhilspgsr (active site)
	dnhsqllvntksgkv-gt (N-terminal)
IV67	tvtifg
II63	dglddivgdhnvicplmhf
II64	vphandlgl(dw)avt
VI64	a(i)lqsgsp(ns)cpwatv-va
IV62-64	fgbgtyly(f)(f)n(h)r
IV18	tgnpnep(p)vzeq
IX02	gpha-a
IV64	aigag(a)(v)ae(pg)g-(v)-ppd
II54	lg(v)(p)s(la)a--(dv)
I109	vdl(l)
II59	lgvpda----d--vp
VI64	a-lqsg(s)pncpw
I109	(te)vdl(l)
II59	lgvpda----d--vp
VI64	a-lqsg(s)pncpw
II67	lgvphad-dg
VI64	ailqsgsp(ds)cpwatv-la

Table 1 Sequences of Torpedo californica Acetylcholinesterase Peptides (continued)

11S Acetylcholinesterase (CNBr peptides)

bt1	mddnngiknrdglddivgdhnvicp
bt2	m-wfg-p-pepgkpwngv
bt3	mlntgnfkk-qillgvn-fgif-lyga(v)(g)f
bt4	mhvwatfaktgnpnep-eg--kwplifik-(fq)-(e)
bd1	mwnpdre(p)
bd2	mnrvsnyypfgpgvyflsieapd

*Roman numerals denote Sephadex fractions of the initial fractionation and Arabic numbers denote the peak fraction from high-pressure liquid chromatography. CM-cysteine denotes radioactivity associated with the carboxymethyl group, indicating that the peptide may contain cysteins; carbohydrate indicates carbohydrate-containing peptides. Parentheses indicate uncertain sequences and dashes indicate an undetectable amino acid appearing at that position. Peptides lacking identification codes were refractionated to achieve greater purity prior to sequencing.



Sequence analyses of the active site tryptic peptides and chymotryptic peptide. The *arrow* indicates the [^{3}H]isopropylphosphoryl-labeled serine. The consensus sequence is indicated in *circles* at the *top*. Residues in *parentheses* indicate placement only by amino acid composition. Residues indicated by *capital letters* are positions identified unambiguously, whereas sequences in *lower case letters* were tentatively identified.

Figure 1: Active center sequences of the 11S and 5.6S acetylcholinesterases.

has been labeled with monobromobimane in the 5.6S enzyme. Isolation and sequencing of the peptide reveals that the cysteine is at position 231

Sites of glycosylation have been identified by lectin blotting of the individual peptides and by broad elution profiles that reflect microscopic heterogeneity within the peaks and their coalescence following endoglycosidase F treatment. An example is shown in Fig. 2. Three of the four potential N-linked glycosylation sites have been located by peptide isolation (asparagine positions 59, 457, and 533), while it appears that position 416, despite the presence of an Asn, X, Ser/Thr, is not glycosylated. Overall carbohydrate compositions suggest that we may have an O-linked site, but this remains to be established.

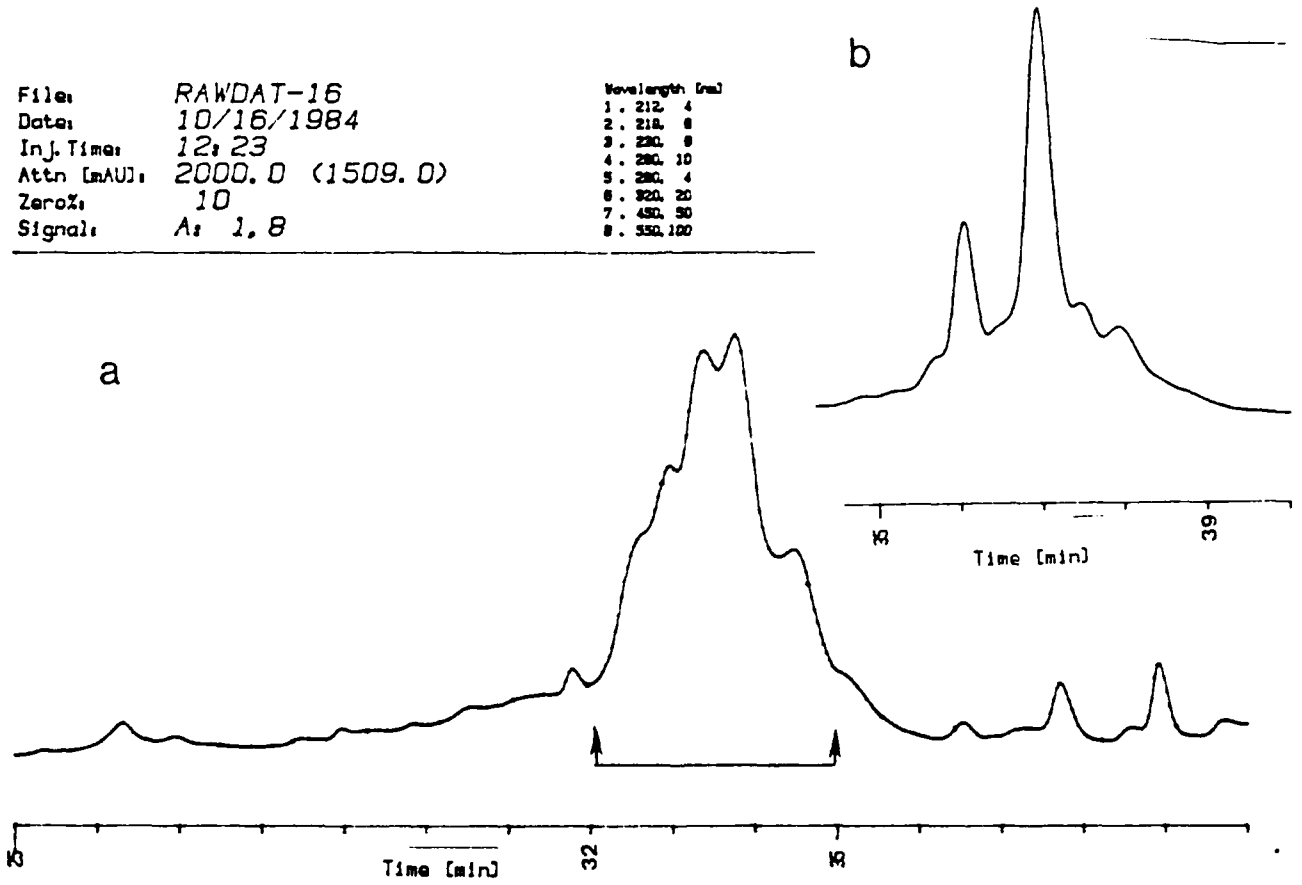


Figure 2: HPLC profiles of 11S acetylcholinesterase peptides prior to (a) and following (b) endoglycosidase F treatment. Fractions 32-35 were isolated, lyophilized and treated with endoglycosidase F. The fractions were run on the same column (C-18 reverse phase), using an identical trifluoroacetic acid-H_2O-acetonitrile gradient. The elution profile with its altered elution positions and decreased complexity is shown in the inset (b).

d. Potential sites that serve as epitopes for the monoclonal antibodies raised by B.P. Doctor (10) have also been identified. The two of particular interest are 4E-7 and AE-2. 4E-7 reacts selectively with the hydrophobic 5.6S enzyme (2) and has been found to react only with the glycosylated form of the enzyme. Treatment with endoglycosidase F but not endoglycosidase H eliminates the antigenicity. 4E-7 reacts equally well with the native and denatured enzyme. A peptide extending between residues 358 and 386 shows the greatest reactivity with 4E-7 as determined by antibody blotting and competitive immunoprecipitation. We expect this peptide to be one of those unique to the 5.6S enzyme.

The other antibody of interest is AE-2, an antibody isolated by Fambrough and colleagues (14) which shows considerable species cross-reactivity. AE2 was found to react with a peptide found by B.P. Doctor in fetal calf serum acetylcholinesterase. This peptide has been found between positions 12 and 18 and considerable homology between species exists in a large portion of this peptide (Fig. 3). Several other antibodies are less well characterized. However, some, such as 4G-7 and 2C-9, show high titers and good immunoprecipitation capacity.

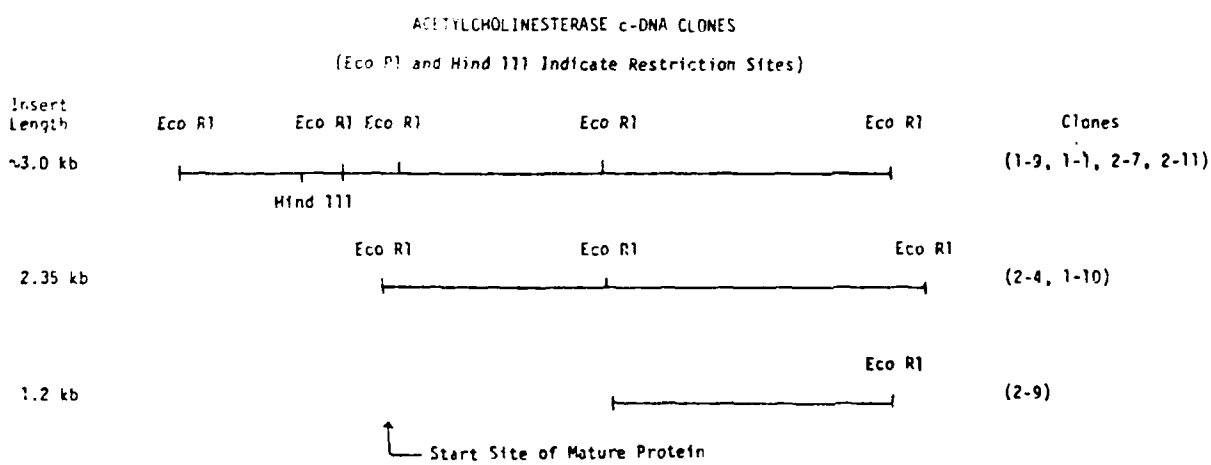


Figure 3: Characterization of several clones encoding for acetylcholinesterase. The length of the clones and their Eco R1 and Hind III sites are shown.

The rather brief description given here describes the bulk of the studies performed during the past 2 years. Extensive fractionation and sequencing were required to achieve this state of progress for an enzyme subunit size of 575 amino acids which exists in multiple enzyme forms. These endeavors have been very much facilitated by the instrumentation provided in the contract. Profiles of some of the many fractionations can be found in MacPhee-Quigley et al. (9).

2. Preparation of antibodies directed to the active center for acetylcholinesterase

Having obtained the active center peptide sequence, we then synthesized a 25-mer peptide to generate antibodies to the active center of acetylcholinesterase. The peptide was synthesized by the Merrifield solid-phase method, using Dr. Russell Doolittle's facility, Department of Chemistry, University of California, San Diego. An N-terminal lysine was added to promote solubility, giving the sequence:

Lys-Thr-Val-Thr-Ile-Phe-Gly-Glu-Ser-Ala-Gly-Ala-Ser-Val-Gly-Met-Ile-Leu-Ser-Pro-Gly-Ser-Arg.

Antibodies are being generated in three ways: Monoclonals are being raised by B.P. Doctor and M.K. Gentry at Walter Reed Army Institute of Research. The fusions are now complete, positive colonies have been selected, and we should be screening for precise titers and selectivity next month. Polyclonal antibodies are also being raised in rabbits at San Diego, using two forms of immunogens: the peptide dispersed in liposomes and the peptide conjugated to hemocyanin. The monoclonal antibodies have the potential of being directed to small peptidic domains, some of which will show little species cross-reactivity. Others, owing to extensive homology in the epitope, will exhibit considerable species cross-reactivity. The polyclonal antibodies can be expected to have the higher titers and will prove most useful for screening in vitro translation products and the development of highly sensitive assays for the active center of acetylcholinesterase. Antibodies to synthetic peptides have the advantage of not showing cross-reactivity to contaminant proteins in biological preparations.

3. Comparative sequencing of Torpedo and other cholinesterases

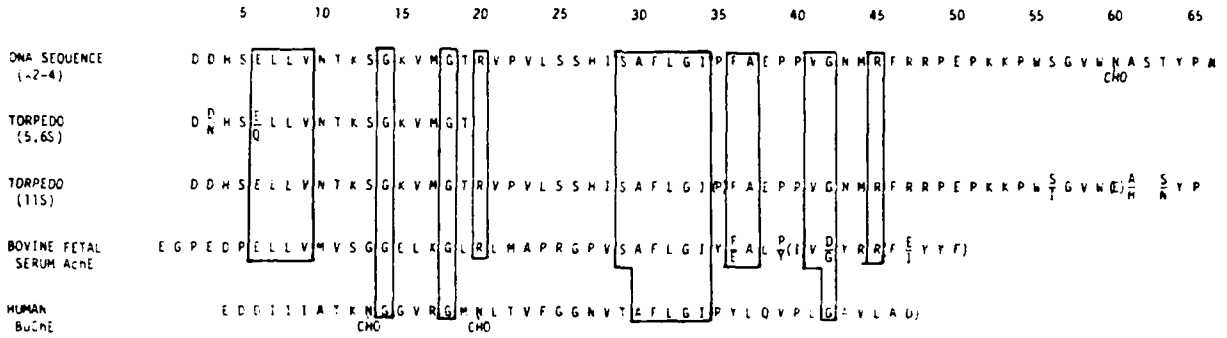
Our initial findings showing extensive homology of the active center peptides of Torpedo acetylcholinesterase and human butyrylcholinesterase (Table II) and the substantial homology in the N-terminal region of the two proteins (Table III) prompted a further homology search in conjunction with Drs. Oksana Lockridge and Bert LaDu at the University of Michigan, and we see extensive similarity throughout the two molecules. Several peptides showing corresponding sequences can readily be found if our peptides (Table I) and their peptides are compared. The Michigan group also has about 80% of the peptides sequenced and with a total inferred sequence available in Torpedo, it should be possible for them to place the remaining peptides within the linear sequence (15).

Table II Sequences of Active Site Regions

	5 P 10 15 20
TORPEDO ACETYLCHOLINESTERASE	NH_2 - THR VAL THR ILE PHE GLY GLU SER ALA GLY GLY ALA SER VAL GLY MET HIS ILE LEU SER
EEL ACETYLCHOLINESTERASE	GLY GLU SER SER GLU GLY ALA ALA GLY
HUMAN PSEUDOCHOLINESTERASE	NH_2 - SER VAL THR LEU PHE GLY GLU SER ALA GLY ALA ALA SER VAL SER LEU HIS LEU LEU SER
EQUINE PSEUDOCHOLINESTERASE	PHE GLY GLU SER ALA GLY SER ALA ALA
EQUINE ALIESTERASE	PHE GLY GLU SER ALA GLY ALA ALA SER
BOVINE TRYPSINOGEN	LYS ASP SER CYS GLN GLY ASP SER GLY GLY PRO VAL VAL CYS SER GLY LYS
PORCINE TRYPSIN	LYS ASP SER CYS GLN GLY ASP SER GLY GLY PRO VAL VAL CYS ASN GLY GLN
S. GRICEUS TRYPSIN	VAL ASP THR CYS GLN GLY ASP SER GLY GLY PRO MET PHE ARG LYS ASP ASN
E. COLI ALKALINE PHOSPHATASE	LYS PRO ASP TYR VAL THR ASP SER ALA ALA SER ALA THR ALA TRP SER THR

Human butyrylcholinesterase and Torpedo acetylcholinesterase can be expected to diverge on a phylogenetic basis and the basis of distinct enzymatic properties (i.e., the butyrylcholinesterase will accommodate substrates with large acyl groups, it does not show substrate inhibition and it is preferentially inhibited by different alkylphosphates). Therefore, one might expect that other mammalian acetylcholinesterases will possess structures showing structural divergence between these two limiting cases. In this regard, the fetal bovine serum acetylcholinesterase has proven useful. The trend in sequence divergence that we might expect can be seen in examining the N-terminal region of four cholinesterases (Table III). A more complete analysis of this nature should prove very useful in identifying various functional and antigenically cross-reactive regions.

Table III N-Terminal Sequences of the Cholinesterases



The bovine fetal acetylcholinesterase sequence is from B.P. Doctor (unpublished results). Human butyrylcholinesterase was reported in reference 15.

4. Isolation of c-DNA clones encoding for acetylcholinesterase

Although this portion of the work was initiated and sustained with the support of the National Institutes of Health, the protein chemistry and molecular biological approaches are integrally linked, and it would have been impossible to proceed as rapidly without having both approaches in the same laboratory. Our library screening strategems rely on hybridization with tandem but not overlapping probes, since we initially found that screening with a single probe yielded a very high incidence of false positives. The library was constructed in a γgt-10 vector and kindly provided by Dr. Toni Claudio at Yale University, New Haven, Connecticut. When sequenced, the false positives were found to be repeating sequences of approximately 500 bp with rather good base matches (14 of the 17 bases in the mixed probe). The tandem probes eliminated this artifact and were preferable to using probes coding for separate peptides. The latter approach will miss short-length sequences. The tandem probe approach usually requires that more amino acid sequence be known, since rather long peptide stretches are usually required to minimize code redundancy in the probes. Positives to both tandem probes were then screened with a probe coding for the N-terminal region. This reduced the number of positives and enhanced the likelihood of obtaining full-length inserts. By this approach we have now obtained 7 inserts which clearly encode for acetylcholinesterase and 13 more candidates. Their lengths and locations of Eco R1 sites are detailed in Fig. 3. Only lambda 2-4 has been sequenced. The sequence and sequencing strategies are given in Figs. 4 and 5.

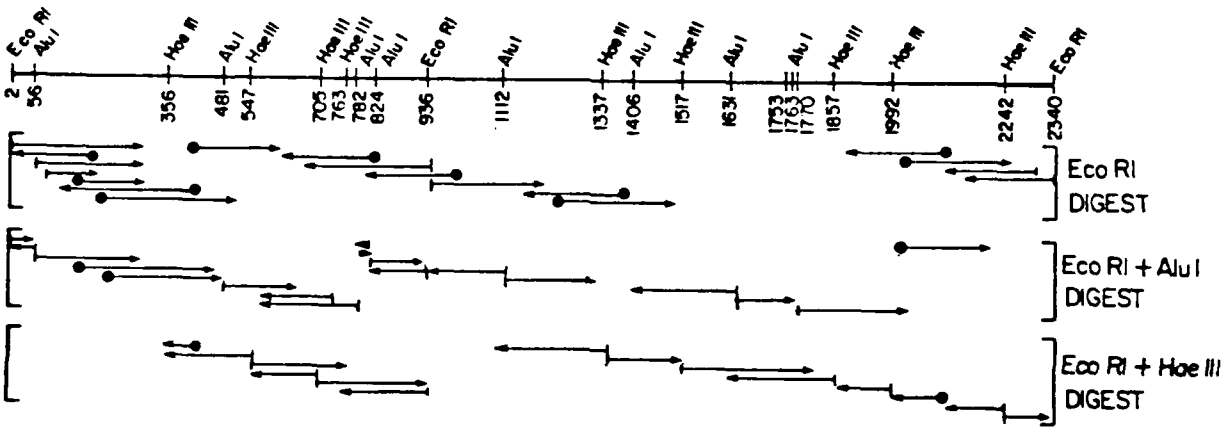


Figure 4: Sequencing strategy for acetylcholinesterase clone AchE-1 (λ2-4). Critical restriction sites (Eco R1, Hae III, Alu 1) and sequencing primer sites are shown.

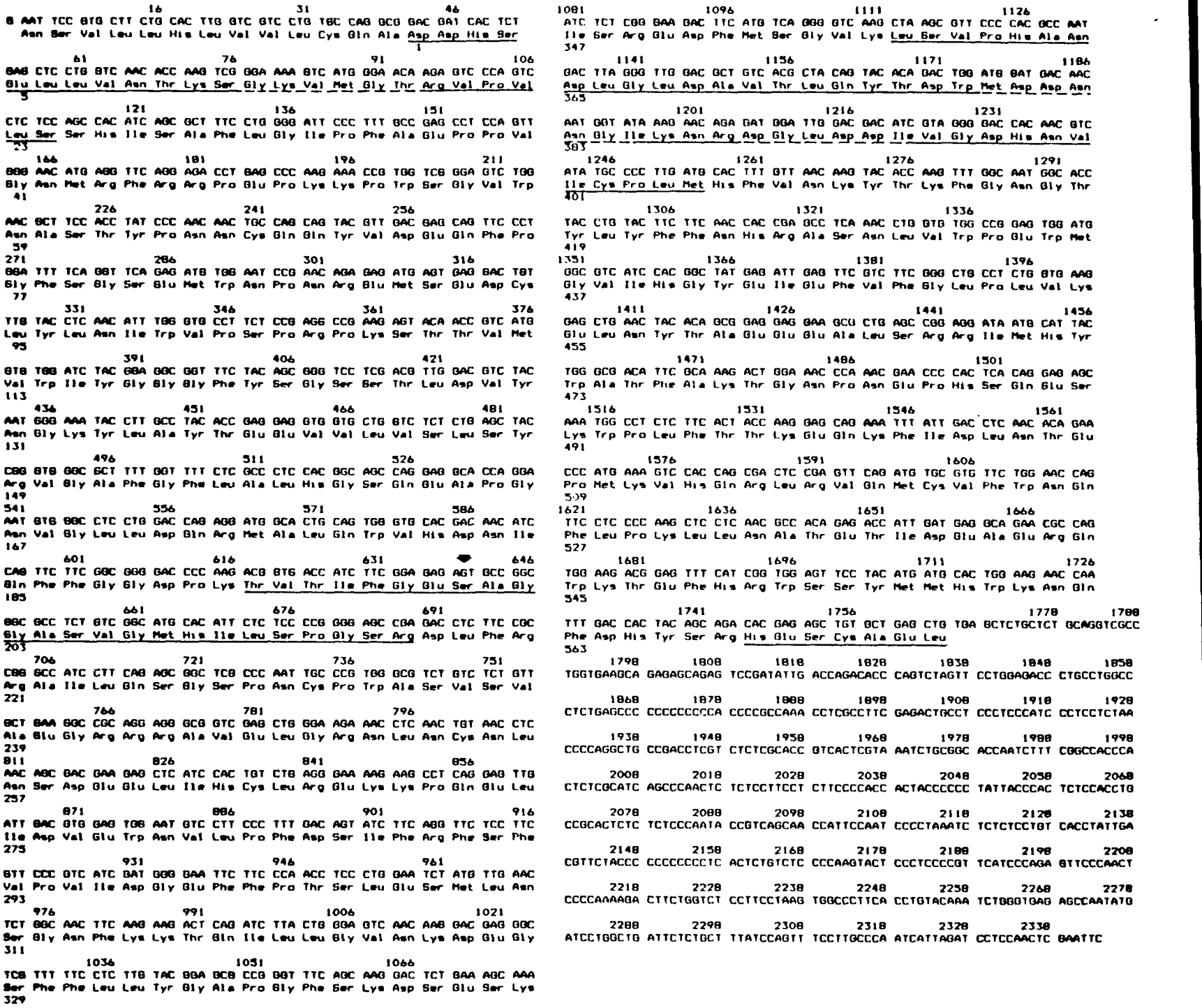


Figure 5: c-DNA and inferred amino acid sequence of clone 2-4.

The insert begins by encoding a 16-amino acid leader peptide, extends through bases encoding the 575 amino acids of the processed protein, and contains another 571 site bases in a 3' noncoding region (fig. 5). It does not contain a poly A termination site or a canonical poly A termination signal, which suggests that this 3' region is not complete (4). Clone 2-4 was sequenced in M-13 by the dideoxy method. Protein sequence, again, provided confirmation that the selected open reading frame was correct and did not divaricate because of a skipped base. In addition, the protein sequence enabled us to establish that the clone likely encoded for the 11S species. We are sequencing the other clones based on initial findings, and are optimistic that we have found a clone for another acetycholinesterase species. There is one caveat: The fact that all clones end in Eco R1 sites suggests incomplete methylation in the library preparation. Clones 1-1, 1-9 and 1-10 are probably identical but reflect another gene of acetylcholinesterase. They are being sequenced. Clone 1-9, which is nearly 2.9 kb in length, is our candidate for obtaining complete 5' and 3' noncoding regions. Clone 2-9 is probably a shortened version of 1-9, terminating at the Eco R1 site. In short, valuable information will continue to accrue as we compare c-DNA inferred sequences with actual protein sequences. Accordingly, the combination of molecular biology and protein chemistry should enable us to identify all of the structural polymorphisms in Torpedo acetylcholinesterase.

5. Isolation of additional acetylcholinesterase clones

The original clone (AchE-1) was used to select additional clones in the λ-gt-10 cDNA library. In all, 117 positive recombinants were identified and isolated. Since the library was amplified, all of the recombinants do not reflect unique inserts, but on the basis of insert size we have been able to identify at least 15 unique clones. Unfortunately, none of the clones encode a sequence we can identify as the 5.6S species. This has also been confirmed with initial data where we have employed a probe which spans both sides of the putative exon-intron exon splice site which gives rise to the message for the 11S enzyme.

Nevertheless, three clones yielding unique information have been identified:

a. AchE-11. This clone is identical in the open reading frame to AchE-1, but contains an extended 5' region, giving us the entire 21 amino acid leader sequence and a sequence of approximately 140 base pairs in the 5' noncoding region.

b. AchE-14. This clone is identical in the open reading frame to AchE-1 and AchE-11 but contains a deletion in the 5' noncoding region. This appears to be an alternative sequence in this region. The deletion point starts at a bona fide splice site.

c. AchE-16. This clone, while short, extends the sequence of AchE-1 in the 3' direction for another 80 bp.

6. Determination of the secondary structure of acetylcholinesterase

A major portion of the third year was directed to this important project. The procedures involved tryptic and CNBr digestion of the unreduced protein followed by separation of the individual peptides on HPLC. Peptides not detected in the reduced enzyme profiles were separated. They were reduced and carboxymethylated with [^{14}C]iodoacetate.

The reduced peptides were analyzed for radioactivity and sequence. Once free, SH could be detected by labeling with monobromobimane in the absence of reduction. This was Cys ^{231}. Three pairs of disulfide-bonded peptides were identified: loop A, Cys^{67}-Cys^{94}; loop B, Cys^{254}-Cys^{265}; loop C, Cys^{402}-Cys^{521}. Cys ^{572} was linked to an identical peptide. This disulfide, three residues from the C-terminal position, forms the intersubunit cross-link with homologous subunits. The three intrachain disulfide loops A, B and C are conserved in thyroglobulin and butyrylcholinesterase, demonstrating that these proteins are likely to have identical folding patterns. The disulfide bonding pattern and homology with thyroglobulin are shown in Figs. 6 and 7.

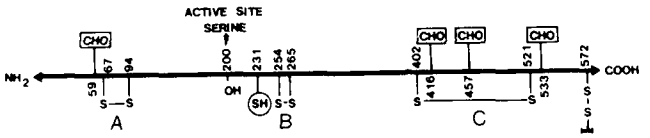


Figure 6. Localization of the sulfhydryl groups and glycosylation sites in acetylcholinesterase. CHO indicates carbohydrate attachment sites, disulfide bonds are connected by solid lines (S-----S), OH indicates the active site serine, and SH indicates a free sylfhydryl group; NH_2 and COOH indicate the amino- and carboxy termini, respectively. The three disulfide loops are designated A, B, and C. Numbers denote the position of each residue based on the cDNA-deducted sequence.

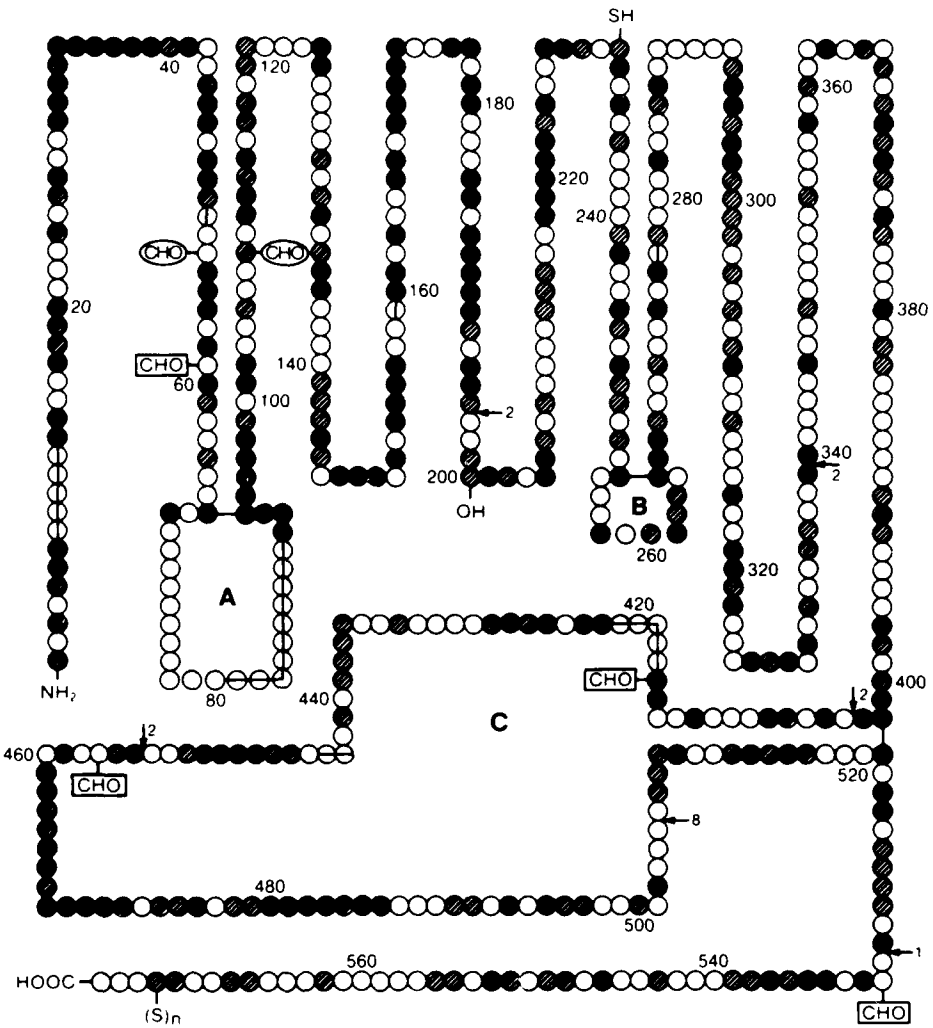


Figure 7

Schematic representation of the homology and common secondary structure between acetylcholinesterase (residues 1–575) and thyroglobulin (residues 2182–2750). Numbering is based on the deduced sequence of acetylcholinesterase. Loops created by disulfide bonds are indicated by *A*, *B*, and *C*. ● indicates identical residues; ○ indicates conservative changes; *OH* indicates the active site serine; *SH*, the free sulfhydryl group; CHO, AChE carbohydrate attachment sites; $(S)_n$, disulfide link to a secondary AChE monomer. Homologous residues with thyroglobulin are as indicated. *Arrows* denote residue gaps in acetylcholinesterase; the *number* indicates the number of residues missing; ~ indicates residue gaps in thyroglobulin sequence. (CHO), thyroglobulin carbohydrate attachment sites. Conservative changes reflect amino acid substitutions that could be encoded by a single base difference.

7. General aspects of acetylcholinesterase structure deduced from amino acid and nucleotide sequencing

All of the above data enable us to arrive at the following conclusions:

a. Acetylcholinesterase contains a hydrophobic leader sequence (residues -21 to 0) but contains no other hydrophobic domains which are candidates for membrane-spanning regions. Thus it is likely to be an exported protein and its membrane attachment site(s) arises as a consequence of posttranslational modifications.

b. The active center serine is at residue 200. The N-terminal location contrasts with the serine proteases of similar size that function in the clotting cascade (i.e., factor IX and prothrombin).

c. No significant global or local homology is found with the acetylcholine receptor.

d. Although acetylcholinesterase is closely homologous to human butyrylcholinesterase, no significant global homology and very limited local homology are found with other serine proteases: The largest local homology is seen with liver aliesterase and the carboxylesterases.

e. Substantial homology is found between acetylcholinesterase and thyroglobulin in their C-terminal regions (acetylcholinesterase residues 1-575; thyroglobulin residues 2168-2750). Six of the eight cysteines are conserved, suggesting a similar folding pattern for the two macromolecules. The region between residues 160 and 190 shows greater than 60% identity.

f. The homology with thyroglobulin shows that six conserved cysteines are the ones forming the three disulfide loops in acetylcholinesterase. The SH involved in the intersubunit disulfide linkage at 572 and the free cysteine Cys 231 are not conserved. This striking comparison shows that the secondary structure and folding pattern of acetylcholinesterase and thyroglobulin are virtually identical.

g. The homology between Torpedo acetylcholinesterase and human butyrylcholinesterase (53% residue identity) (15) reveals substantial conservation among the cholinesterases. A comparison of Torpedo acetylcholinesterase with a fetal bovine serum acetylcholinesterase shows about 60% residue identity. The latter enzyme is being sequenced by B.P. Doctor and colleagues. Substantial homology ($\sim$30% residue identity) with certain other esterases (microsomal esterase, Drosophila Est 6 and Dictyostelium D-3) have also been reported.

8. Ongoing studies

Several studies have been initiated but results are still too preliminary to give a detailed account. These studies include:

a. Detection of antigenic regions within the acetylcholinesterase molecule. For this purpose several monoclonal antibodies previously mentioned are being employed.

b. Genomic cloning of acetylcholinesterase.

c. Detection of regions within the primary structure in the hydrophobic dimer forms of acetylcholinesterase which are unique and differ from the asymmetric forms.

d. Analysis of carbohydrate structures of the various forms of acetylcholinesterase.

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